sites of 2-APB, we examined the effect of 2-APB incubation on SOCe mediated by these mutants and other reported mutations that could affect functional coupling between STIM1 and Orai1. Surprisingly, none of these mutations prevent the inhibition caused by 2-APB preincubation, indicating more critical residues are needed for functional STIM1-Orai1 coupling. Interestingly, one single mutation in the pore of Orai1, Orai1V102C, abolishes the ability of 2-APB to inhibit SOCe acutely. Thus, 2-APB may inhibit SOCe through its actions on the pore region of Orai1 channels.

1607-Pos Board B337

Orai3 TM3 Point Mutation G158C Alters Kinetics of 2-Apb-Induced Gating by Disulfide Bridge Formation with TM2 C101

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After endoplasmic reticulum (ER) Ca^{2+} store depletion, Orai channels in the plasma membrane (PM) are activated directly by ER-resident STIM proteins to form the Ca^{2+}-selective Ca^{2+} release-activated Ca^{2+} (CRAC) channel. However, in the absence of Ca^{2+} store depletion and STIM interaction, the mammalian homolog Orai3 can be activated by 2-aminoethyl diphenylborinate (2-APB), resulting in a nonselective cation conductance characterized by biphasic inward and outward rectification. Here, we use site-directed mutagenesis and patch-clamp analysis to better understand the mechanism by which 2-APB activates Orai3. We find that point mutation of glycine 158 in the third transmembrane (TM) segment to cysteine, but not alanine, slows the kinetics of 2-APB activation and prevents complete channel closure upon 2-APB washout. Orai3-G158C channels are trapped in an open state, as indicated by a lack of recovery upon 2-APB washout. The ''slow'' phenotype exhibited by Orai3 mutant G158C reveals distinct open states, characterized by reversal potentials that shift gradually from 47 to 7 mV during activation. The slow phenotype can be reversed by application of the reducing reagent bis(2-mercaptoethylsulfone) (BMS) in a state-dependent manner, only during 2-APB activation. Moreover, the double mutant C101G/G158C, in which an endogenous TM2 cysteine is changed to glycine, does not exhibit altered kinetics of 2-APB activation. We suggest that a disulfide bridge, formed between the introduced cysteine at TM3 position 158 and the endogenous cysteine at TM2 position 101, hinders transitions between Orai3 2-APB-induced open and closed states. Our data provide functional confirmation of the proximity of these two residues and suggest a location within the Orai3 protein that mediates activation and gating by 2-APB.

1608-Pos Board B338

Interplay of Orai1-Loop3 with Extracellular Ca^{2+} Binding Sites in Loop1 Controls Crac Channel Activity

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Ca^{2+} release-activated Ca^{2+} (CRAC) channels are a major pathway for Ca^{2+} entry in a vast majority of cell types. The identification of the ER Ca^{2+} sensor STIM1 and the plasma membrane channel subunit Orai1 lead to a substantial progress in our understanding of molecular structures underlying the unique permeation properties of CRAC channels. Nevertheless, Ca^{2+} coordination at the pore entrance and the role of the extracellular loop1 and loop3 of hOrai1 proteins still remain rather unclear.

Based on a recent crystal structure of the drosophila Orai channel we generated an all-atom model of human Orai1 including the missing extracellular loops. Using a combined approach of patch-clamp, cysteine-crosslinking and molecular dynamics simulations we investigated the pore entrance of hOrai1 channels. Our experiments showed that the extracellular loop3 of hOrai1 exhibited a high degree of crosslinking along the whole loop compatible with a remarkable flexibility. Crosslinking studies between loop1 and loop3 demonstrated close proximity, suggesting a movement of loop3 affecting the pore entrance. Molecular dynamics simulations indicated that Ca^{2+} ions bind to the extracellular aspartates located in the first loop. Further, an interaction of the third loop with these negatively charged amino acids seems to compete with the Ca^{2+} ions, thereby reducing CRAC channel activity. Sequence alignment of Orai1 isoforms identified that the essential residues for hOrai1 loop1-loop3 coupling are conserved in higher mammals.

With this work we suggest a pivotal/regulatory role of the third loop in modulating hOrai1 function. Via movement and subsequent coupling of loop3 to aspartates in the first loop it may regulate the Ca^{2+} sink at the pore entrance and thus permeation through CRAC channels.

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1609-Pos Board B339

Novel Trans-Membrane Mutation Switches Orai1 to a Constitutively Active and Ca^{2+} Selective Channel

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The endoplasmic reticulum Ca^{2+} sensor STIM1 forms together with the Ca^{2+} channel Orai1 the molecular basis for Ca^{2+} release activated Ca^{2+} (CRAC) channels. The recent crystal structure of Orai from Drosophila melanogaster shows a unique Ca^{2+} channel composed of a hexameric subunit complex. The pore structure is formed by transmembrane (TM) 1 helices, surrounded by two ring-like structures, formed by TM2 and TM3 as well as TM4. Employing a combined approach of patch-clamp, molecular biology, biochemical techniques, molecular modeling and structure guided mutagenesis; we discovered a novel key mutation in the second trans-membrane helix of Orai1 that results in a Ca^{2+} selective, STIM1 independent, constitutively active current. Substitution of this essential residue to a hydrophobic amino-acid retained store-operated activation, yet with largely reduced Orai1 currents. In addition, we took advantage of the constitutively active Orai1 mutant, to evaluate reorientation of the gate located within the cytosolic region of TM1 helices. Cysteine scanning mutagenesis within the TM1 helix enabled identification of gating residues, the dimerization of which was altered in the constitutively opened and closed Orai1 channel conformation. In molecular dynamic simulations of an all-atom model of human Orai1 we will show the interaction network of these identified residues and predict implication of mutations on conformational changes. Our experiments will be summarized in a unique gating model, and we will moreover discuss how STIM1 binding might trigger the open channel conformation. This work was supported by the Austrian Science Foundation (FWF): P26067 to R.S. and P25172 to C.R. Irene Frischauf is an Elise Richter Scholarship holder: V286.

1610-Pos Board B340

Atomistic Molecular Dynamics Simulations of Drosophila Orai in a Hydrated Lipid Bilayer

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Orai channels are prominent (calcium) Ca^{2+} signal mediators in many cell types. In T lymphocytes, Orai1 and STIM1 form the Ca^{2+} release-activated Ca^{2+} (CRAC) channel. Following endoplasmic reticulum (ER) Ca^{2+} store depletion, ER membrane-resident Ca^{2+} sensor STIM1 physically interacts with plasma membrane-bound, pore-forming Orai1 subunits. The STIM1-Orai1 interaction results in Ca^{2+} influx and ultimately changes in gene expression that underlie the immune response. In 2012, the Drosophila Orai (dOrai) crystal structure was solved to 3.34 Å resolution. To develop an atomistic view of Orai channel function, given the high sequence identity (73%) between dOrai and human Orai1, we constructed a complete dOrai structure by building in flexible loops missing in the crystal structure and adjusting the protonation state of several pore residues. The model was placed into a lipid bilayer with excess hydration and simulated for over 200 ns. We find shifted pKa values for several titratable residues in the pore interior, and that changing protonation states of these key residues is essential to maintain pore stability. To explore the mechanism of channel block, we also simulated the model with either Ca^{2+} or (gadolinium) $Gd3^{+}$ in the selectivity filter. Taken together, our results help to address the stability of the closed structure in a lipid bilayer while also yielding novel atomistic insights into channel block and pH sensitivity.

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1. Hou, X, et al. Science. (2012); 338; 1308-13.

1611-Pos Board B341

Orai3 Dominantly Modulates Redox Sensitivity and Requires Orai1 to Localize to Microdomains of Store-Operated Activation

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Plasma-membrane localized Orai1 ion channel subunits interacting with ER localized STIM1 molecules comprise the major subunit composition responsible for calcium release-activated calcium (CRAC) channels. Mammals also express the Orai1 homologs Orai2 and Orai3 in most cells albeit with differential stoichiometry. While Orai2 and Orai3 have been described as bona fide store-operated channels when overexpressed, Orai3 is also an essential subunit of non-store operated channel complexes activated by arachidonic acid, estrogen or thrombin. In $CD4^{+}$ T cells, Orai3 modulates oxidation mediated

inhibition of store-operated calcium entry (SOCE). Here we use patch-clamp analysis, TIRF and confocal microscopy, Ca^{2+} imaging and biotinylation experiments to systematically investigate whether Orai2 or Orai3 are capable of forming true store-operated or store-independent channels by themselves or whether and when they require Orai1. Using Jurkat T cells stably expressing Orai homologs, we track localization to the immunological synapse, determine redox sensitivity of SOCE and using concatenated constructs, unravel the stoichiometric requirements for redox inhibition of heteromeric channels. Our results demonstrate a dominant requirement for the presence of Orai1 to escort and confer store-operated activity onto Orai3 subunits and vice versa a dominant requirement for Orai3 to yield redox insensitive heteromeric SOCE complexes with Orai1.

1612-Pos Board B342

Icrac in Human Primary Prostate Epithelial Cells

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Expression levels of membrane androgen receptors (mAR) are elevated in prostate cancer and correlate with a negative prognosis. Rapid androgen response in prostate cancer can counteract several cancer hallmark functions such as unlimited proliferation, enhanced migration, adhesion and invasion and the inability to induce apoptosis. Thus mAR have been proposed as targets for therapeutic strategies. However, the molecular identity and downstream signaling pathways of mAR are still elusive. In primary cultures of human prostate epithelial cells from non-tumorous tissue (hPEC), we identified a rapid 5alpha-dihydrotestosterone (DHT) induced activation of store-operated Ca^{2+} entry.

1613-Pos Board B343

Activation of STIM1 by L-Glutamate Rapidly Inhibits L-Type Calcium Channel Current in Cultured Hippocampal Neurons

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At glutamatergic synapses, synaptic activity elicits postsynaptic Ca^{2+} signals that are generated in part by voltage-gated, L-type calcium channels. L-channel Ca^{2+} signals are known to control transcription of genes involved in synaptic plasticity, such as during late-phase long-term potentiation. Here we report that, in short-term cultured rat hippocampal neurons, 15-sec application of L-glutamate inhibited pharmacologically-isolated L-type Ca^{2+} current: inhibition ensued with a $t_{1/2}$ of 49 $\pm$ 2 sec, and current was reduced by ~35%. Measurements made with a genetically-encoded Ca^{2+} indicator (D1ER) targeted to intracellular stores showed that application of L-glutamate triggered release of Ca^{2+} from stores. FRET-imaging demonstrated that L-glutamate application also induced interaction of the Ca^{2+} store-depletion sensor STIM1 (CFP-tagged) with the L-channel scaffolding protein, AKAP79/150 (YFP-tagged), but not another AKAP scaffold protein, AKAP15/18. The time course of L-channel inhibition paralleled that for the increase in STIM1-AKAP79/150 FRET, suggesting a functional relationship between glutamate-triggered STIM1 translocation and inhibition of L-channel current. Supporting this idea, RNAi knockdown of STIM1 abolished glutamate-induced inhibition of L-channel activity. The ability of glutamate to inhibit L-channels in STIM1-knockdown neurons was rescued by co-transfection of rat neurons with the human STIM1 isotype, which was insensitive to the rat RNAi. Our results suggest a model wherein (i) glutamate receptor activation triggers Ca^{2+} release from endoplasmic reticulum (ER) stores within seconds of glutamate application, (ii) lowered ER Ca^{2+} activates STIM1 translocation to ER/plasma membrane junctions, and (iii) STIM1 interaction with the L-channel-AKAP79/150 complex down-regulates L-type Ca^{2+} current. This STIM1-mediated link between glutamate receptors and L-type Ca^{2+} channels may tune cytoplasmic Ca^{2+} signals involved in synaptic plasticity of hippocampal neurons.

1614-Pos Board B344

Characterization of Store-Operated Calcium Channels in Pancreatic Duct Epithelia

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Store-operated Ca^{2+} channels (SOCs) are activated by depletion of intracellular Ca^{2+} stores following agonist-mediated Ca^{2+} release. In pancreatic duct epithelial cells (PDEC), the activation of GPCR coupled to phospholipase C stimulated SOC-mediated Ca^{2+} influx. Direct measurement of $[Ca^{2+}]$ in the ER showed that SOCs slowed ER depletion. SOC-mediated currents were inwardly rectifying and greatly increased in the absence of divalent cations, as typical for SOCs in other cell types. Pharmacology of epithelial SOCs was consistent with that of some types of SOCs. In polarized PDEC, SOCs were localized specifically to the basolateral membrane. Both STIM and Orai proteins were expressed in PDEC and were colocalized after store depletion. Furthermore, knockdown of Orai3 expression, the most abundant Orai subtype, reduced SOC-mediated Ca^{2+} influx significantly. In conclusion, basolateral Ca^{2+} entry through SOCs fills internal Ca^{2+} stores depleted by external stimuli and facilitates Ca^{2+}-dependent cellular processes such as salt and mucin secretion from the exocrine pancreatic ducts.

1615-Pos Board B345

Cholesterol Regulates Orai1 Function

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STIM1 and Orai proteins represent the essential molecular components of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Following ER-store depletion, CRAC current activation occurs due to the physical interaction between the STIM1 C-terminus and Orai1 N- and C-termini. Here we focused on an additional role of the extended transmembrane Orai1 N-terminal (ETON) region in cholesterol binding as it contains a cholesterol binding motif. Both chemically induced cholesterol depletion as well as point mutations disrupting the Orai1 cholesterol binding site enhanced store-operated Orai1 currents about 2-fold. Currents were not increased due to enhanced plasma membrane expression as revealed by biotinylation experiments. In addition, Orai1 point mutants that disrupted the cholesterol binding motif were not anymore sensitive to chemically induced cholesterol depletion. In accordance, employing intrinsic fluorescence measurements we detected direct binding of cholesterol to an N-terminal fragment containing the cholesterol binding motif with an equilibrium dissociation constant (K_D) of about 2μM, while mutations disrupting it increased the K_D 3-5 fold compared to wild-type. In aggregate, we propose a modulatory role of cholesterol on CRAC channel function. (supported by the Austrian Science Fund: FWF project P25210 to I.D., FWF project M01506000 to I.J. and FWF project P25172 to C.R.)

1616-Pos Board B346

Orai and TRPC Channel Contribution to Calcium Signaling in Human Mast Cells

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Mast cells are important targets for the treatment of allergic diseases and anaphylaxis. Inappropriate and chronic activation of mast cells via the IgE receptor leads to the release of a range of pre-stored and newly synthesized inflammatory mediators and the symptoms of disease. Calcium influx is a critical regulator of mast cell signaling, with influx through ion channels absolutely required not only for the exocytosis of preformed mediators but also to direct the synthesis of eicosanoids, cytokines and chemokines. Studies in rodent and human mast cells have identified STIM-regulated Orai channels to be key players in initiating calcium influx and degranulation in antigen-stimulated mast cells; in rodent mast cells, a role for TRPC channels is also emerging. Here we report evidence for a role of TRPC channels in human mast cell signaling. Using gene microarray analysis, we find evidence for expression of multiple TRP family members in primary human lung mast cells and LAD2 cells; the expression of TRPC1 and TRPC6 was further confirmed by immunocytochemistry. Single cell fura-2 imaging experiments, showed that barium could partially substitute for calcium to support influx following antigen- and thapsigargin stimulation, consistent with a contribution of non-selective TRPC-like channels to store operated calcium entry in human mast cells. To investigate the potential involvement of TRPC1 in STIM-regulated store operated calcium signaling in human mast cells, LAD2 cells were transfected with the STIM1 (684KK685) TRPC1 gating mutant. Expression of the mutant, however failed to alter calcium signaling in either thapsigargin or antigen-stimulated cells. Further pharmacological and molecular experiments are currently being performed to further evaluate the putative role of other TRPC channels in human mast cell signaling.